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1. Kim et al Cancer Research 1986 November Vol 46 (11) : 5985-92
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Differential Expression of Difucosyl Type 2 Chain (Le^Y) Defined by Monoclonal Antibody AH6 in Different Locations of Colonic Epithelia, Various Histological Types of Colonic Polyps, and Adenocarcinomas¹

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ABSTRACT

The expression of Le^Y (Fuc α 1 \rightarrow 2Gal β 1 \rightarrow 4[Fuc α 1 \rightarrow 3]GlcNAc β 1 \rightarrow R) (in which Fuc is fucose, Gal is galactose, and GlcNAc is N-acetylglucosamine) defined by monoclonal antibody AH6 in various locations of human colonic epithelia, colonic polyps, and adenocarcinomas has been studied. In normal colonic mucosa, strong staining by AH6 was observed in the proximal regions such as the terminal ileum and cecum. The staining was, however, greatly reduced in the epithelia of the ascending colon and became very weak in the epithelia of transverse, descending, and sigmoidal colon as well as the rectum. Of 481 crypts in 40 biopsy samples of the epithelia of normal sigmoidal colon, 421 crypts did not show any staining, and only a weak staining of the lowest crypt area was observed in 60 crypts (12%); of 474 crypts in 40 biopsy samples of normal rectal epithelia, 110 crypts (26%) showed a weak staining. At the fetal stage, the Le^Y staining was much more intense in all locations of the colon than in corresponding locations of adult epithelia, and staining was observed in the epithelia of the sigmoidal colon and rectum. A strong staining was observed in 24 of 25 cases of colorectal adenocarcinomas, irrespective of their original location. The expression of Le^Y in polyps was correlated with histological type as well as the degree of dysplasia of the polyps. Of various adenomas examined, tubular adenomas, many of which showed mild or moderate dysplasia and less malignant potentials, displayed the least Le^Y expression. Tubulovillous and villous adenomas, which have higher malignant potential and showed a higher incidence of severe dysplasia, showed a greater area and intensity of Le^Y expression. No Le^Y was detectable in juvenile polyps, and only a very weak staining was observed in the dysplastic area of hyperplastic polyps. The extent and intensity of staining in various adenocarcinomas and adenomas could not be correlated with blood group ABO status of the hosts nor with location of the tumors. These results suggest that Le^Y in colonic adenocarcinomas and polyps at the distal region of the colon and rectum is a typical oncofetal antigen and is a useful marker for diagnosis of colonic cancer. Its expression in colonic polyps can be correlated with the degree of dysplasia and may indicate the degree of malignant potential of the polyp. Thus, Le^Y expression in polyps may have prognostic value.

INTRODUCTION

Increasing evidence obtained by chemical analysis and by examination with monoclonal antibodies has shown that many of the tumor-associated antigens in human gastrointestinal tumors are modified blood group antigens (for reviews, see Refs. 1 and 2). Typical examples are Le^{x2} (3-7), di- or trimeric Le^a (8, 9), their sialylated derivatives (10, 11), and Le^Y (12-15). Expression of these antigens is oncofetal, i.e., they are maximally expressed at certain stages of embryogenesis and organogenesis, regress greatly after development is complete, and are relatively restricted in their distribution in adult gastrointestinal tissues (16, 17). Some of these markers, such as sialosyl Le^a ,

sialosyl Le^x , and sialosyl dimeric Le^a , may be released into plasma of patients with cancer, and the levels of these antigens have been found to be of diagnostic value (18, 19, 20). We (12) and others (13, 14) have previously established monoclonal antibodies directed to Le^Y , which were obtained after immunization with cell lines from gastric cancer (12), lung cancer (13), and colonic cancer (14). Expression of this antigen in various cases of colonic cancers and normal colonic mucosa has been previously described (15). However, distribution of this antigen in various locations of normal colonic mucosa in comparison with colonic cancers from different locations and colonic polyps of various histological types and with various degrees of dysplasia had not been studied in detail. This paper focuses on expression of the antigen in normal fetal and adult colonic epithelia and in various types of colonic polyps with different degrees of dysplasia and malignant potentials.

MATERIALS AND METHODS

Tissue Samples. Various cases of colonic cancers were obtained from surgical operations performed at the Department of Surgery, Osaka Medical College, Hirakata City Hospital, and Ohno Hospital, Osaka, Japan. Colonic polyps were obtained from endoscopic polypectomy performed at the Second Department of Internal Medicine, Osaka Medical College. Twelve biopsy samples from various locations in colon, rectum, and terminal ileum, as shown in Fig. 1, were obtained by colonofiberscopic examination of three patients with no signs of lesions of the colon. Other biopsy samples of the epithelia of normal sigmoidal colon and normal rectum were obtained by sigmoid fiberoscopy from 40 normal cases (2 biopsy samples were obtained from each case). Seven fetal colons were obtained from autopsy of fetuses at 24, 25, 26, 28, 30, 35, and 38 wk of gestation. All the samples were fixed in neutral buffered formalin, embedded in paraffin, dissected into 5- μ m sections, and examined by the immunohistological procedure described below.

Immunohistological Examination of Tissue Sections. Immunoperoxidase staining of histological samples was performed by the avidin-biotin-peroxidase complex method (21) using Vectastain ABC Kit (Vector Laboratories Inc., Burlingame, CA). Tissue sections were deparaffinized and treated with methanol containing 0.3% hydrogen peroxide for 10 min at room temperature in order to block the activity of endogenous peroxidase. Subsequently, sections were incubated with normal horse serum diluted 1:150 with saline, which prevents nonspecific binding of the sections to the antibody. The culture supernatant of monoclonal antibody AH6 (12), in chemically defined media without fetal calf serum, was diluted 10 times. The sections were incubated for 2 h, followed by reaction with biotinyl anti-mouse horse serum, avidin, and biotinyl-peroxidase complex according to the manufacturer's directions. The sections were developed in phosphate-buffered saline containing 0.02% diaminobenzidine and 0.005% hydrogen peroxide and counterstained with Mayer's hematoxylin.

Glycolipid Analysis. In order to further confirm the presence of Le^Y glycolipids in colonic adenocarcinoma at the distal region of colon (adenocarcinoma derived from transverse colon material) and the absence of Le^Y glycolipids in the distal region of normal colonic epithelia,

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² The assignments Le^a and Le^Y are adopted for the structures previously designated X and Y (29), respectively, based on the fact that they are positional isomers of blood group Lewis antigens Le^a and Le^b , although Le^a and Le^Y are not a subtype of Lewis antigens.

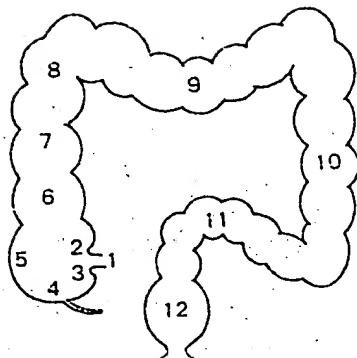


Fig. 1. Locations where biopsy samples were taken by colonofiberscopic examination.

the tissues were extracted and examined by TLC³ immunostaining with monoclonal antibody AH6. Epithelia of normal gastric mucosa were used as a control. Forty-eight g of colonic adenocarcinoma, 19 g of normal colonic mucosa, and 26 g of normal gastric mucosa (antrum) were each extracted with isopropanol/hexane/water, and the upper neutral glycolipid fraction was prepared as previously described (12). Aliquots (10–15 μ g) of the upper neutral glycolipid fraction derived from each tumor were placed on a Baker Si-HPF HPTLC plate (J. T. Baker Chemical Co., Phillipsburg, NJ), developed with chloroform:methanol:water (60:35:8), and immunostained by the peroxidase method as described below. The developed HPTLC plate was first incubated with 1% BSA in phosphate-buffered saline for 1 h at room temperature. The plate was then washed with PBS and incubated with 10-times diluted culture supernatant of AH6 hybridoma for 2 h followed by washing with PBS containing 0.1% BSA. The washed plate was subsequently overlaid with 200-times diluted biotinylated horse antibody directed to mouse immunoglobulin, incubated for 1 h, and washed with PBS containing 0.1% BSA. The plates were further incubated with avidin and biotinylated peroxidase complex (Vector Laboratories) for 30 min and finally washed four times with PBS containing 0.1% BSA. The glycolipid bands immunofixed with peroxidase were developed in the presence of 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA). Glycolipid bands on TLC plates were developed with 2% orcinol in 2 M sulfuric acid.

RESULTS

Location of Difucosyl Type 2 Chain (Le^Y) in Normal Colonic Epithelia. Twelve biopsy samples obtained on colonofiberscopic examination of three patients who had no signs of lesions in the colon were subjected to immunoperoxidase staining. The

ing patterns are shown in Fig. 2, A–E. As indicated in Fig. 2A, all foveolae in colonic epithelia were strongly immunostained by AH6 at the terminal ileum and cecum. The number of foveolae showing positive staining decreased in the epithelia of ascending colon (see Fig. 2B). Staining in the epithelia of the transverse and descending colon was barely visible, except in the basal glandular region. In a separate study, many crypt areas (crypts) in the biopsy samples of normal sigmoidal colon and rectum were compared. No staining was observed in the majority of the biopsy samples examined. As shown in Table 1, of 481 crypts in 40 biopsy samples of sigmoidal epithelia, only 60 crypts (12.4%) showed weak staining and the remaining 421 crypts did not show any staining with AH6. The incidence of staining in the crypts of rectal epithelia was higher (25.9%), but the intensity of the staining was very weak. The majority

(351 of 474) of the crypts in rectal epithelia was not stained. A positive staining of the crypts in the rectum is shown in Fig. 2, D and E.

A similar decrease of staining by AH6 from the proximal to the distal colon was observed in the fetal colon as well, although the intensity of staining in fetal colon was much stronger than that in adult tissues. However, in fetal tissue, positive staining was also observed in the epithelia of the sigmoidal colon and rectum (Fig. 2, F and G). In general, staining of fetal tissues by AH6 was predominantly at the luminal surface. Six other samples of fetal colon tissue showed very similar staining patterns (individual data not shown).

Immunoperoxidase Staining of Adenocarcinomas and Various Types of Colonic Polyps. The expression of Le^Y in colonic adenocarcinomas and various types of polyps is summarized in Table 2. Twenty-four of 25 cases of colonic adenocarcinomas (96%) were positively stained with AH6 antibody. A typical example of staining of adenocarcinoma in contrast to adjacent normal sigmoidal epithelia and rectal epithelia is shown in Fig. 2, H and I. The expression of Le^Y in polyps (adenomas, and hyperplastic and juvenile polyps) can be correlated with both histological type and degree of dysplasia. Of various adenomas examined, most tubular adenomas showed mild or moderate dysplasia (42 of 44 cases), and only a few showed severe dysplasia (2 of 44 cases). Those cases with severe dysplasia showed the highest degree of Le^Y expression. A similar tendency was observed in tubulovillous and villous adenomas, both of which showed a higher malignant potential and higher degree of dysplasia than tubular adenomas. Le^Y expression in both tubulovillous and villous adenomas was much greater in both the extent and intensity of expression than in tubular adenomas, and Le^Y expression can be correlated with malignant potential (see Table 2). In contrast, hyperplastic and juvenile polyps showed a much lower degree of dysplasia than did adenomas; juvenile polyps did not express Le^Y, and hyperplastic polyps with mild dysplastic foci showed a low incidence of Le^Y expression (25%) (see Table 2). The Le^Y expression as correlated with the degree of dysplasia is summarized in Table 3. Staining of Le^Y in a moderately dysplastic area of tubular adenoma and in tubulovillous adenoma is shown in Fig. 2, K and L, respectively.

Correlation between Le^Y Expression and Size and Location of Polyps. The incidence and degree of Le^Y expression appeared to be correlated with the size of the polyps (Table 4). Le^Y was more strongly expressed in larger than in smaller polyps. All

of which were, however, tubulovillous or villous adenomas with moderate or severe dysplasia. In juvenile and hyperplastic polyps, Le^Y was not expressed or was only weakly expressed regardless of their size (various cases with diameters ranging from 5–18 mm examined; data not shown). There were no differences in Le^Y expression between polyps located in the sigmoidal and rectal colon (Table 4).

Correlation of Intensity of Le^Y Expression in Tumors with Location of Tumor and Host's Blood Group Status. The intensity of staining by AH6 of adenocarcinomas and adenomatous polyps varied according to the degree of dysplasia. As shown in Table 5, more intense staining was associated with adenocarcinomas than with adenomas with various degrees of dysplasia. Tissues of adenomatous polyps with mild dysplasia showed a much lower incidence of staining than did those with moderate or severe dysplasia; however, within a given polyp containing severe dysplasia, the areas showing severe and moderate dysplasia were stained to nearly the same degree of AH6. The correlation between the intensity of staining by AH6 and the

³ The abbreviations used are: TLC, thin-layer chromatography; BSA, bovine serum albumin; PBS, phosphate-buffered saline (10 mM sodium phosphate, pH 7.0 and 0.9% sodium chloride); HPTLC, high-performance thin-layer chromatography.



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Table 1 Expression of Le^y in crypts of normal sigmoidal colon and rectum (biopsy samples)

Location	No. of crypts examined	No. of crypts stained by antibody AH6	% of crypts stained by antibody AH6
Sigmoidal colon	481	60	12.4
Rectum	474	123	25.9

Table 2 Expression of Le^y in colonic adenocarcinoma and in polyps with particular focus on histological types and degree of dysplasia of adenomas and hyperplastic polyps

	No. studied	No. stained by antibody AH6	% stained by antibody AH6
Adenocarcinoma	25	24	96.0
Polyps			
Adenoma	57	28	49.1
Tubular	44	15	34.1
Mild dysplasia	30	8	26.7
Moderate dysplasia	12	5	41.7
Severe dysplasia ^a	2	2	100.0
Tubulovillous	10	10	100.0
Moderate dysplasia	5	5	100.0
Severe dysplasia ^a	5	5	100.0
Villous	3	2	66.7
Moderate dysplasia	2	1	50.0
Severe dysplasia ^a	1	1	100.0
Hyperplastic polyps	12	4	25.0
No dysplasia	8	0	0.0
Mild dysplasia	4	2	50.0
Juvenile polyps	4	0	0.0

^a The histological area showing severe dysplasia was less than 30% of the entire area; the rest of the area showed moderate dysplasia.

Table 3 Correlation between Le^y expression and degree of dysplasia in adenomas

Degree of dysplasia	No. studied	No. stained by antibody AH6	% stained by antibody AH6
Mild	29	8	27.6
Moderate	20	11	55.0
Severe	8	8	100.0

Table 4 Size and location of polyps and their Le^y expression

	No. studied	No. stained by antibody AH6	% stained by antibody AH6
Diameter of polyps			
3-9 mm ^a	38	13	34.2
10-19 mm ^a	26	12	46.2
>20 mm ^b	5	5	100.0
Location of polyps			
Sigmoidal colon	51	21	41.2
Rectum	18	0	0.0

^a Eighty % of cases were tubular adenomas and 20% were tubulovillous or villous adenomas. In tubular adenomas, Le^y was expressed in 20% of polyps with diameters 3-4 mm, 50% with diameters 7-8 mm, and 80% with diameters 9-11 mm; however, only 30% with diameters 12-18 mm were stained.

^b Four of 5 polyps larger than 20 mm had a portion of severe dysplasia, and they were all tubulovillous or villous adenomas.

location of adenocarcinomas is shown in Table 6. In contrast to the intensity of staining of normal colonic epithelia, which was greater at the proximal region and decreased at the distal region, the intensity of staining of adenocarcinomas in distal and proximal colon was similar.

Since the expression of Le^y in human erythrocytes from blood group O individuals was found to be much stronger than the expression in erythrocytes from blood groups A and B individuals (12), the intensity of staining with AH6 in various adenocarcinomas from different blood group types was compared. The results are shown in Table 7. The intensity of antigen expression could not be correlated with the host's blood group ABO status.

Glycolipid Profile. To confirm the immunohistological re-

Table 5 Degree of staining by antibody AH6 in adenocarcinomas and adenomas

	Degree of staining						Total
	++++	+++	++	+	±	-	
Adenocarcinoma	5	7	5	7	0	1	25
Adenoma							
Mild dysplasia	0	0	1	6	2	20	29
Moderate dysplasia	0	0	3	6	1	9	20
Severe dysplasia							
Portion of severe dysplasia	0	0	3	4	1	0	8
Portion of moderate dysplasia	0	0	2	5	1	0	8

Table 6 Correlation between intensity of staining by AH6 and original location of adenocarcinomas

Original location of adenocarcinomas	Intensity of staining					Total
	++++	+++	++	+	-	
Cecum	1	4		3		8
Ascending colon				1		1
Transverse colon				1		1
Descending colon	1					1
Sigmoid colon	2		2	2	1	7
Rectum	1	3	3			7

Table 7 Correlation between intensity of staining by AH6 and ABO blood group of host patient with colonic cancer

Blood group	Intensity of staining					Total
	++++	+++	++	+	-	
O	1	2	2	3	0	8
A	2	4	2	2	1	11
B	1	1	1	0	0	3
AB	1	0	2	0	0	3

sults, which demonstrated clear differences between adenocarcinomas of the distal region of colon and their normal counterparts, glycolipids of colonic cancer derived from transverse colon and normal parts of colonic mucosa as well as normal gastric mucosa (antrum) were studied. The results are shown in Fig. 3. Three bands of Le^y were clearly stained by orcinol-sulfuric acid (lane 1) and by AH6 (lane 4) in the upper neutral glycolipid fraction of adenocarcinomas; whereas the glycolipid bands from the distal region of normal colonic mucosa, which migrated faster on HPTLC, were not stained (lanes 2 and 5). The same glycolipid fraction derived from normal gastric mucosa showed a different TLC pattern and gave only a faint single band stained by AH6 antibody (lanes 3 and 6).

DISCUSSION

Several studies have indicated that colorectal cancer and polyps express modified blood group-related antigens defined by monoclonal antibodies and lectins (14-17, 22-25). These markers are quantitatively different from those of normal colonic mucosa. Since colonic polyps with certain histological characteristics can be regarded as premalignant lesions (26), and in particular, dysplasia of tissue (27) can be correlated with premalignant potential, any markers characteristic of premalignant polyps are of great clinical value.

We have previously established a hybridoma secreting monoclonal antibody AH6 (12). The hybridoma was established after immunization with human gastric cancer cell line MKN74 and was selected by a preferential reactivity with human cancer cell lines over various types of other human cell lines. The antibody was found to define difucosylated type 2 chain (Le^y; Fucal→2Galβ1→4[Fucal→3]GlcNAcβ1→3Gal). Two other monoclonal antibodies displaying similar specificities have been described by Brown *et al.* (13) and Lloyd *et al.* (14). These

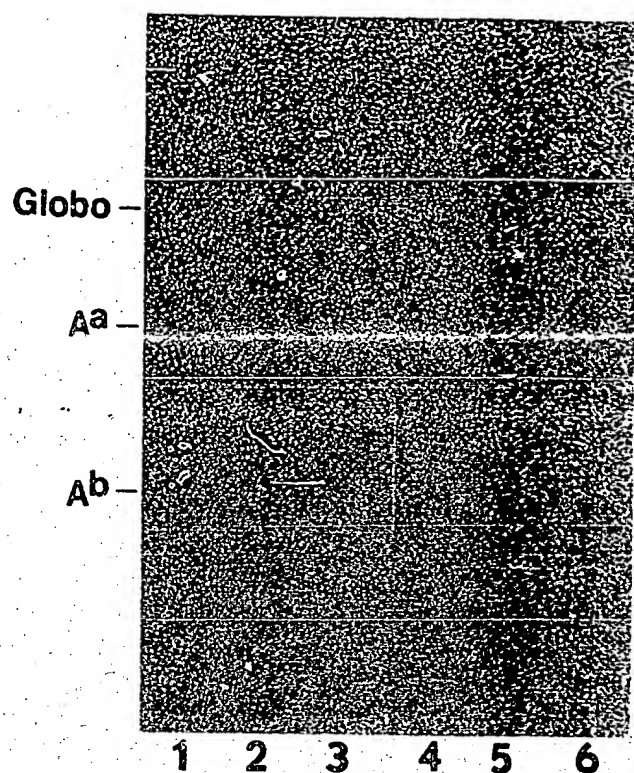


Fig. 3. Thin-layer chromatogram of upper neutral glycolipids isolated from human colonic cancer and gastric mucosa (left) and their immunostaining pattern by AH6 (right). Lanes 1 and 4, glycolipids of human colonic adenocarcinoma tissue; lanes 2 and 5, glycolipids of normal colonic mucosa distant from cancer tissue of the same patient as lanes 1 and 4; lanes 3 and 6, glycolipids isolated from normal gastric mucosa of duodenal ulcer patient. Lanes 1-3 were stained by orcinol-sulfuric acid reaction, and lanes 4-6 were stained by avidin-biotin-peroxidase complex method. Globo, globoside, GalNAc α 1 \rightarrow 3Gal β 1 \rightarrow 4Gal β 1 \rightarrow 4Glc β 1 \rightarrow ICer, A^x, ceramide hexasaccharide, GalNAc α 1 \rightarrow 3[Fuca1 \rightarrow 2]Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 3Gal β 1 \rightarrow 4Glc β 1 \rightarrow ICer, A^y, ceramide octasaccharide, GalNAc α 1 \rightarrow 3[Fuca1 \rightarrow 2]Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 3Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 3Gal β 1 \rightarrow 4Glc β 1 \rightarrow ICer.

antibodies were established after immunization with human colonic adenoma and human lung cancer cell lines, respectively. More recently, Brown *et al.* (15) described immunohistological studies of colonic adenocarcinomas and adenomas with different blood types, although they did not study the distribution of Le^y in various locations of normal colonic epithelia and in various types of colonic polyps as correlated with blood types and degree of dysplasia. A systematic study of colonic polyps is of great clinical importance, since some types of colonic polyps can be regarded as premalignant lesions (26). This paper is therefore focused on (a) localization-dependent differences of Le^y expression in normal colonic mucosa and (b) a systematic comparison of Le^y expression in adenocarcinomas and various types of polyps and the correlation with their locations, histological variation, and degree of dysplasia. The results of the present study clearly indicate that: (a) expression of Le^y in normal colonic mucosa depends greatly on the location. Less staining by AH6 antibody was observed in the distal than in the proximal region of colonic mucosa, and no staining was detectable in the sigmoidal colon and rectum in the majority of crypts in the biopsy samples. A limited number of crypts (60 of 481 from sigmoidal epithelia and 123 of 474 from rectal epithelia) was weakly stained at the basal cells of the crypts by AH6 antibody; (b) fetal colonic epithelia were stained much more strongly by AH6 antibody than adult epithelia, although the intensity of staining in fetal colonic epithelia also decreased in the distal region. The fetal sigmoidal colon and rectum were

positively stained by AH6, in contrast to the lack of staining in adult sigmoidal colon and rectum; (c) essentially all adenocarcinomas (greater than 96%) expressed Le^y, regardless of their origin and blood type; (d) a close correlation between Le^y expression and the degree of dysplasia, which is associated with the histotypes of polyps, was observed. Juvenile polyps, which do not show dysplasia and have no malignant potential, did not express Le^y, and hyperplastic polyps with mild dysplasia showed a very weak Le^y expression, whereas tubulovillous and villous adenomas, which often show severe dysplasia and have high malignant potential, strongly expressed Le^y. Tubular adenomas, which are essentially benign polyps, had no dysplasia and expressed little Le^y; (e) polyps of large size, which contain more dysplastic area than smaller polyps, showed greater Le^y expression. On the other hand, no correlation could be found between the location of polyps and Le^y expression.

Since Le^y expression in the distal region of fetal colon was much stronger than in the corresponding region of adult epithelia, the antigen must be a typical oncofetal antigen in colorectal adenocarcinoma and polyps. The incidence of Le^y expression in adenomas in this study was much lower than the previous results reported by Brown *et al.* (15); furthermore, the incidence of Le^y expression could not be correlated with blood group ABO status, in contrast to the results of the study by Brown *et al.* (15). These discrepancies must be due to differences in antibody characteristics. Affinities and reactivities of various antibodies directed to Le^x hapten (Gal β 1 \rightarrow 4[Fuca1 \rightarrow 3]GlcNAc β 1 \rightarrow 32Gal β 1 \rightarrow R) and their analogues (multimeric Le^x) vary extensively, although the antibody reactivity can be inhibited by the same oligosaccharide sequence (11, 28). The antibody showing a preferential reactivity to dimeric Le^x shows a more restricted staining of normal tissue but much greater staining of tumor tissue than antibodies directed to simple monomeric-Le^x (11, 25). A systematic comparison of immunohistological staining of colonic cancers with various antibodies directed to Le^x and its analogues showed clear differences (25).

Of particular clinical interest is the staining by AH6 antibody of 100% of polyps with severe dysplasia, which is often associated with tubulovillous and villous adenomas, as compared to the low incidence of staining of polyps with mild or no dysplasia, which is associated with tubular adenomas (26.8%), hyperplastic polyps (25%), and juvenile polyps (0%). Since tubulovillous and villous adenomas are considered to have greater malignant potential than tubular adenomas and hyperplastic and juvenile polyps (26), positive reactivity with AH6 antibody may have prognostic value for colonic polyps.

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